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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,194,814**, on January 10, 1997, by **THE JOHN P. ROBARTS RESEARCH INSTITUTE**, assignee of Terry L. Delovitch, for "Stimulation of Protective T Cells to Prevent Autoimmune Disease"

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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Stimulation of Protective T Cells to Prevent Autoimmune Disease

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Notice: This application is as filed and may therefore contain an incomplete specification.

STIMULATION OF PROTECTIVE T CELLS TO PREVENT AUTOIMMUNE DISEASE

This invention relates to methods and compositions for preventing the development of autoimmune disease in susceptible subjects. More particularly, the invention relates to treatment with an agonist of CD28 to prevent autoimmune disease development.

Background of the Invention

Insulin-dependent diabetes mellitus (IDDM) or autoimmune diabetes is a polygenic, multifactorial, autoimmune disease heralded by T cell infiltration of the pancreatic islets of Langerhans (insulitis) and the progressive T cell-mediated destruction of insulin-producing β cells (Bach, 1994; Atkinson and Maclaren, 1994; Tisch and McDevitt, 1996).

Non-obese diabetic (NOD) mice are susceptible to the development of IDDM and are an accepted model for the development of autoimmune IDDM in humans.

$CD4^+$ T helper cells are required for the adoptive transfer of IDDM into recipient neonatal NOD mice or immunodeficient NOD.Scid mice (Bendelac et al., 1987, Christianson et al., 1993; Rohane et al., 1995). Cooperation between $CD4^+$ and $CD8^+$ T cells is required to initiate IDDM, and islet β cell destruction is $CD4^+$ T cell-dependent (Haskins and McDuffie, 1990; Wang et al., 1991). Current evidence suggests that the $CD4^+$ effector cells of IDDM in NOD mice at Th1 cells which secrete IL-2, IFN-γ and TNF-α and that the regulatory $CD4^+$ cells are Th2 cells which secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Rabinovitch, 1994; Liblau et al., 1995; Katz et al., 1995).

NOD mouse T cells show proliferative hyporesponsiveness to T cell receptor (TCR) stimulation

and this hyporesponsiveness may be causal to the development of IDDM.

It has been shown that, beginning at 3-5 weeks of age, T cell receptor (TCR) ligation in NOD mice induces the proliferative hyporesponsiveness of NOD thymic and peripheral T cells, which is mediated by reduced IL-2 and IL-4 production (Zipris et al., 1991; Rapoport et al., 1993a; Jaramillo et al., 1994).

Decreased IL-4 production by human T cells from patients with new onset IDDM has also been demonstrated recently (Berman et al., 1996). Whereas addition of IL-4, a Th2-type cytokine, potentiates Il-2 production and completely restores NOD T cell proliferative responsiveness, addition of IL-2, a Th1-type cytokine, even at high concentrations, only partially restores NOD T cell responsiveness. These findings suggest that Th2 cells may be compromised in function to a greater extent than Th1 cells in NOD mice, and raise the possibility that Th2 cells require a higher threshold of activation than Th1 cells in these mice. Il-4 not only restores NOD T cell responsiveness *in vitro*, but prevents insulitis and IDDM when administered *in vivo* to prediabetic NOD mice (Rapoport et al., 1993a) or when transgenically expressed in pancreatic β cells (Mueller et al., 1996).

The proliferative hyporesponsiveness of regulatory Th2 cells in NOD mice may favour a TH1 cell-mediated environment in the pancreas of these mice, and lead to a loss of immunological tolerance to islet β cell autoantigens. This is consistent with the notion that restoration of the balance between Th1 and Th2 cell function may prevent IDDM (Rabinovitch, 1994; Liblau et al., 1995; Arreaza et al., 1996).

Optimal T cell activation requires signalling through the TCR and CD28 costimulatory receptor (June et al., 1994; Bluestone, 1995; Thompson, 1995). Crosslinking of the TCR/CD3 complex in the absence of a

CD28-mediated costimulatory signal induces a proliferative unresponsiveness that is mediated by the inability of T cells to produce IL-2 (Jenkins et al., 1991). CD28 costimulation prevents proliferative unresponsiveness in Th1 cells by augmenting the production of IL-2, which in turn promotes IL-4 secretion by T cells (Seder et al., 1994). The costimulatory pathway of T cell activation involves the interaction of CD28 with its ligands B7-1 and B7-2 on an antigen presenting cell (APC), with B7-2 considered as the primary ligand for CD28 (Linsley et al.,1990; Freeman et al., 1993; Lenschow et al., 1993; Freeman et al., 1995). When costimulation is blocked by either CTLA4-Ig or by anti-B7-1 or anti-B7-2 monoclonal antibodies (mAbs), differential effects on the incidence of various autoimmune diseases (e.g. IDDM) and on the development of Th1 and Th2 cells are observed (Kuchroo et al., 1995; Lenschow et al., 1995). Furthermore, *in vivo* studies have demonstrated that the generation of Th2 cells is more dependent upon the CD28-B7 pathway than the priming of Th1 cells, and suggest that the development of Th subsets *in vivo* may be influenced by limited CD28-B7 costimulation (Corry et al., 1994; Lu et al., 1994). Analyses of the development of human Th2 cells have yielded results similar to those observed in the mouse (King et al., 1995; Kalinski et al., 1995; Webb and Feldman, 1995). Interactions between CD28 and its B7-2 ligand are essential for the costimulation of an IL-4-dependent $CD4^+$ T cell response, and IL-4 increases B7-1 and B7-2 surface expression on certain professional APCs (eg. Langerhans cells) and B cells (Freeman et al., 1995; Kawamura et al., 1995; Stack et al., 1994). Thus, failure to activate NOD thymocytes and peripheral T cells sufficiently may be due to functional and/or differentiation defects in NOD APCs, which remain able to optimally activate islet β cell autoreactive $CD4^+$ effector

T cells, but not regulatory CD4$^+$ T cells (Serreze et al., 1988; Serreze et al., 1993). Functional defects that compromise antigen presentation by NOD APCs, such as deficient CD28 costimulation, may lower their ability stimulate regulatory Th2 cells without compromising their ability to stimulate autoreactive effector Th1 cells.

Proliferative hyporesponsiveness of T cells has been observed in other autoimmune diseases such as multiple sclerosis and myasthenia gravis.

If proliferative hyporesponsiveness of T cells in autoimmune disease could be overcome, it might be possible by that means to prevent the development of autoimmune diseases.

Summary of Drawings

Certain embodiments of the invention are described, reference being made to the accompanying drawings, wherein:

Figure 1A shows thymocyte proliferation, and Figure 1B shows splenic T cell proliferation, expressed as ^{3}H thymidine incorporation in cpm, in the presence (circles) or absence (squares) of various concentrations of anti-CD28 monoclonal antibody.

Solid symbols : BALB/c mice

Open symbols : NOD mice

Figure 1C shows IL-2 production and Figure 1D shows IL-4 production, expressed as ^{3}H thymidine incorporation into CTLL-2 and CT.4S cells respectively, by NOD (open symbols) or BALB/c (solid symbols) thymocytes activated by anti-CD3 in the presence (circles) or absence (squares) of 1 μg/ml anti-CD28 MAb.

Figure 2 shows insulitis scores in 8 week-old NOD mice (Panel A) and 25 week-old NOD mice (Panel B) treated with anti-CD28 antibody or hamster Ig (control).

Figure 3A shows diabetes incidence (%) at various ages in NOD mice treated at age 2 to 4 weeks with anti-CD28 antibody (▲) or control hamster Ig (■).

Figure 3B shows diabetes incidence (%) at various ages in NOD mice treated at age 5 to 7 weeks with anti-CD28 antibody (▲) or control hamster Ig (■).

Figure 4, upper panel, shows IL-4 production (pg/ml) by thymocytes, splenic T cells and islet infiltrating cells isolated from anti-CD28 antibody-treated (solid bars) or control (open bars) NOD mice (8 weeks or 25 weeks of age) in the presence or absence of 145-2C11 anti-CD3ε mAb.

Figure 4, lower panel, shows IFN-γ production (pg/ml) by cells as described for Figure 4, upper panel.

Figure 5 shows proliferation (expressed as incorporation of ^{3}H-thymidine in cpm x 10^{-3}) of thymocytes, splenic T cells or islet infiltrating cells from 8- or 25-week old, anti-CD28 antibody-treated NOD mice, in response to anti-CD3ε antibody.

Stimulation indices (SI) were calculated as the ratio of average cpm of anti-CD3 stimulated cultures/average cpm of control cultures, and are shown in parentheses. Values (mean cpm ± SD) shown are representative of three separate experiments.

Figure 6A shows pancreatic level of IL-4 and IFN-γ (ng cytokine/mg tissue) in NOD mice treated with anti-CD28 antibody (solid bars) or control hamster Ig (open bars).

Figure 6B shows serum levels of IgG1 and IgG2a isotype anti-GAD_{67} antibodies in anti-CD28 antibody-treated NOD mice and controls.

Figure 7 shows the incidence of autoimmune diabetes in NOC.Scid mice injected with splenic T cells from anti-CD28 antibody-treated NOD mice (solid symbols) or control

NOD mice (open symbols) at various times after injection (transfer) of splenic T cells.

Description of the Invention

The present invention provides a method for preventing the development of an autoimmune disease in a susceptible subject by treating the subject with an agonist of the T cell CD28 co-receptor. Autoimmune diseases preventable by this method include IDDM, multiple sclerosis and myasthenia gravis.

In accordance with a preferred embodiment, development of an autoimmune disease is prevented by treating a susceptible subject with an antibody to the CD28 co-receptor.

Human subjects susceptible to the development of IDDM may be identified by screening based on a subject's HLA genetic make-up (Undlien et al., (1997)) or based on detection of predictive serum autoantibodies such as anti-insulin or anti-GAD antibodies (Verge et al., (1996)).

Treatment in accordance with the method of the invention should be administered in the neonatal period, from about 6 months to about 2 or 3 years of age. A series of treatments may be required over the 6 month to 2 year period of life.

A monoclonal antibody which specifically recognises human T cell CD28 receptor may be raised in a suitable animal such as a mouse by conventional methods for raising antibodies. Briefly, the mouse is injected with human T cells and a hybridoma producing a monoclonal antibody of the desired specificity is selected and cloned.

Alternatively, antibodies raised against the T cell CD28 co-receptor of a non-human mammal such as a mouse may be used in the methods and compositions of the

invention, in view of the high degree of conservation of amino acid sequence among mammalian CD28 receptors.

Techniques are available and well known to those in the art to prepare humanised antibodies which have a variable region, specific for the CD28 receptor, synthesised in a non-human mammal, combined with a human constant region. Such humanised antibodies may be preferable for treatment of human subjects.

Optimal T cell activation requires signalling through both the TCR and the CD28 costimulatory receptors of the T cell. The T cells of NOD mice have been shown to be hyporesponsive to T cell receptor-stimulation of proliferation.

The present inventor has shown that this hyporesponsiveness of NOD T cells is associated with defective CD28 receptor costimulation.

It has been shown by the inventor that treatment of NOD mice with a CD28 agonist prevented the development of autoimmune diabetes. Treatment of neonatal NOD mice with an anti-CD28 antibody which gave CD28 costimulation completely restored the proliferative responsiveness of NOD thymocytes and peripheral T cells by augmenting their levels of secretion of IL-2 and IL-4. The stimulated increase in IL-4 secretion was predominant.

The antibody treatment effectively prevented the development of destructive insulitis in NOD mice and prevented the expected development of diabetes.

It is postulated that the antigen presenting cell (APC)-derived costimulatory signal transduced by the CD28 receptor on NOD mouse T cells may be insufficient to stimulate optimum T cell activation and that such CD28-signalled activation of IL-4-producing Th2 cells is necessary for protection from IDDM. The work of the inventor suggests that anti-CD28 antibody prevents IDDM in NOD mice by activating the CD28 signalling pathway in

NOD T cells rather than by blocking the interaction between CD28 and its ligand, B7.

Prevention of IDDM by CD28 costimulation may be mediated by the activation of a subset of $CD4^+$ regulatory T cells that confer protection against IDDM. This subset of $CD4^+$ regulatory T cells may be hyporesponsive in NOD mice and may not receive a sufficient amount of the CD28/B7 costimulatory signal required for clonal expansion and effector function in NOD mice.

It has been proposed that precursor $CD4^+$ Th2 cells require a strong initial T-cell stimulation, and that the amount of IL-4 produced is proportional to the magnitude of the initial T cell stimulation. In the absence of CD28 costimulation, the production of IL-4 remains below the threshold required for the optimal development of Th2 cells (Seder and Paul, 1994; Thompson, 1995; Bluestone, 1995). It is of interest that B7-1 and B7-2 ligation of CD28 mediate distinct outcomes in $CD4^+$ T cells. B7-2 costimulation signals naive T cells to become IL-4-producing T cells, and thereby directs an immune response towards Th0 and Th2 cells (Freeman et al., 1995; Kuchroo et al., 1995). B7-1 costimulation seems to be a more neutral differentiative signal, and initiates the development of both Th1 and Th0/Th2 cells. Presumably, B7-2 plays a dominant role in the production of IL-4 due to its early expression during T cell activation (Freeman et al., 1995; Thompson, 1995). Thus, an insufficient or inappropriate signal resulting from a CD28/B7-2 interaction may be delivered to a subset of regulatory $CD4^+$ T cells in NOD mice, and this subset may not differentiate properly into functional IL-4 producing Th2 cells.

The inventor has examined whether anti-CD28 mAb treatment of NOD mice provides the costimulation required for the expansion of and cytokine production by regulatory IL-4 producing Th2-like cells. Figure 4 shows

that anti-CD3 stimulated (*in vitro*) NOD thymocytes obtained at 8 weeks, peripheral splenic T cells obtained at 8 weeks and 25 weeks and islet infiltrating T cells examined at 25 weeks of age produce significantly higher levels of IL-4 compared with the same subpopulations of cells isolated from control mice treated with a hamster Ig. Shortly after termination of treatment with anti-CD28 mAb, thymic and splenic T cells showed a higher basal (no stimulation) production of IL-4 compared to cells obtained from age-matched (8 week-old) control mice. With the exception of higher splenic T cell basal responses in 8 week-old mice, no differences were detected between the proliferative responses of thymocytes, splenic T cells and islet infiltrating cells from 8 and 25 week-old anti-CD28 treated NOD mice and those of the age matched controls (Figure 5). The increase in basal T cell proliferation and IL-4 production may reflect the preferential costimulation of Th2 cells by anti-CD28 treatment *in vivo*. It has been found that anti-CD28 treatment *in vivo* leads to an increased production of IgG1 (which reflects increased IL-4 production by T cells) rather than IgG2a anti-GAD67 antibodies (Figure 6B). Moreover, the total number of splenic lymphocytes was increased about 1.9-fold at 8 weeks of age and 1.7-fold at 25 weeks of age in anti-CD28 treated NOD mice relative to that of control treated mice (data not shown). These findings support the idea that anti-CD28 treatment elicits the expansion and survival of IL-4 producing Th2 cells in NOD mice.

Anti-CD28 treatment did not significantly alter the level of IFN-γ secretion by T cells from 8 week-old NOD mice compared with that observed in age-matched control mice. However, levels of IFN-γ secretion by thymocytes and splenic T cells from 25 week-old anti-CD28 treated NOD mice were markedly reduced in comparison to those levels detected in control mice. These data demonstrate

the long term down regulation of Th1 cell function, which may arise from the preferential activation of Th2 cells induced by CD28 costimulation during the inductive phase of the autoimmune process. The downregulation and/or functional deviation of Th1 cells towards a Th2 cell phenotype by IL-4 is more effective than and dominant over the inhibition of Th2 cell function by IL-12 (Perez et al., 1995; Szabo et al., 1995; Murphy et al., 1996). These results also agree with reports that IFN-γ secreting Th1 cells potentiate the effector phase of insulitis, IFN-γ is directly involved in β cell destruction (Pilstrom et al., 1995; Rabinovitch et al., 1995; Herold et al., 1996; Shimada et al., 1996) and the early differentiation of naïve T cells into Th2 cells is dependent on CD28 signalling (Webb and Feldman, 1995; Lenschow et al., 1996). It is noteworthy that in human T cells, CD28 costimulation is a critical requirement for the development of Th2-like cells but not Th1-like cells, and Th2 cell function remains CD28-independent after the initial costimulation (Webb and Feldman, 1995).

Although anti-CD28 mAb treatment protects from IDDM, this treatment still allows for the development of a non-destructive insulitis. Therefore, this treatment does not interfere with the migration of diabetogenic T cells to the pancreatic islets. Rather, anti-CD28 treatment appears to induce regulatory T cells in the pancreas to suppress islet β cell destruction and progression to overt IDDM. Evidence in support of this is derived from assays of secretion of IL-4 and IFN-γ by infiltrating cells from mice treated with anti-CD28 or control Ig (Figure 4) and from analyses of the levels of expression of these cytokines in the pancreas of anti-CD28 treated NOD mice at 25 weeks of age (Figure 6A). The intra-pancreatic expression of IL-4 was significantly higher in anti-CD28 mAb treated mice, whereas the expression of

IFN-γ remained essentially unaltered in these mice. Committed autoreactive cells, including Th1 cells, may accumulate in pancreatic islets but the functions of IL-4 predominate to inhibit IFN-γ mediated β cell damage.

FACS analyses of the phenotype and surface expression of various cell adhesion molecules in anti-CD28 treated and control NOD mice at 8-25 weeks of age also indicated that anti-CD28 mAb treatment did not interfere with the migration of diabetogenic T cells to pancreatic islets (data not shown). The levels of surface expression of LFA-1, L-selectin and CD44 on the surface of splenic T cells did not suffer significantly between untreated and anti-CD28 treated NOD mice. Similarly, the levels of surface expression of markers of activation such as CD-69, ICAm-1 and B7-2 on B cells were increased only slightly in anti-CD28 treated NOD mice. The T ($CD3^+$):B ($CD19^+$) and CD4:CD8 T cell ratios in NOD mice were not altered by anti-CD28 treatment.

The activation of the $CD4^+$ Th2 cells may arise from the ability of CD28 ligation to sustain the proliferative response and enhance the longer term survival of T cells by the delivery of a signal that protects from apoptosis through the upregulation of survival factors such as Bcl-x_L.

EXAMPLES

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Methods of molecular genetics, protein and peptide biochemistry and immunology referred to but not explicitly described in this disclosure and examples are reported in the scientific literature and are well known to those skilled in the art.

Materials and Methods

Mice NOD/Del mouse colony was bred and maintained in a specific pathogen-free facility. Diabetes incidence among females in NOD colony was 40-50% at 15 weeks of age and 80-90% by 25 weeks. NOD-scid/scid mice generously provided by Dr. L. Shultz (The Jackson Laboratory, Bar Harbor, ME) were bred in the colony and used as recipients in T cell transfer experiments. The age-and sex-matched BALB/c mice used as controls in the *in vitro* T cell proliferation experiments were also bred in the colony.

Anti-CD28 mAb Treatment Either anti-CD28 mAb (50 µg), from supernatants from 37.51 hybridoma cells (provided by Dr. J. Allison, University of California, Berkeley, CA and also obtainable from ATCC, Ma.) secreting hamster anti-murine CD28 mAbs (Gross et al., 1992), purified by protein G affinity chromatography (Pharmacia Biotech, Uppsala, Sweden), or control hamster Ig (50 µg, Bio/Can Scientific, Mississauga, ON) was administered i.p. every other day to female NOD mice (n=20/group, randomized from 10 different litters) from 2- to 4-weeks of age. These mice were then boosted at 5, 7 and 8 weeks of age. Other groups of NOD mice (n=10/group, randomized from 5 different litters) received the same treatment starting at 5 weeks of age. Blood glucose levels (BGL) were measured weekly with a Glucometer Encore (Miles/Bayer, Toronto, ON). Animals with BGL >11.1 mmol/L (200 mg/dl) during two consecutive weeks were considered diabetic.

Histopathology Analysis Mice were harvested periodically during the course of anti-CD28 or control Ig treatment, and pancreatic tissue was removed, fixed with 10% buffered formalin, embedded in paraffin and sectioned at 5 µm intervals. The incidence and severity of insulitis was examined by hematoxylin and eosin staining as well as insulin staining. A minimum of 20 islets from

each mouse were observed, and the degree of mononuclear cell infiltration was scored by two independent, blinded observers using the following ranking: 0-normal, 1-peri-insulitis (mononuclear cells surrounding islets and ducts, but no infiltration of the islet architecture); 2-moderate insulitis (mononuclear cells infiltrating <50% of the islet architecture); and 3-severe insulitis (>50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture). The immunohistochemical detection of insulin was performed using a porcine anti-insulin antibody (Dako Corp., Carpenteria, CA).

Cell Proliferation and Cytokine Secretion

Splenocytes and thymocytes from NOD or control mice were isolated as described in Rapoport et al., 1993. Splenic T cells were isolated on T cell columns (R & D Systems, Minneapolis, N) to a purity of ≥98%, as assayed by FACS analysis of CD3 cell surface expression. Cells (10^6/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM Hepes buffer, 1mM sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mM 2-ME (all purchased from Gibco Laboratories, Grand Island, NY) with plate-bound 145-2C11 anti-CD3ε mAb (1/500 dilution of ascites; hybridoma kindly supplied by Dr. J. Bluestone, University of Chicago, Chicago, IL) in the presence or absence of various concentrations of the 37.51 anti-CD28 mAb. Cells were harvested after either 48 hr (splenocytes and T cells) or 72 hr (thymocytes), and were then assayed for the incorporation of [^{3}H]thymidine (1 μCi/well; Amersham, Oakville, ON) added during the last 18 hr of culture.

Islet infiltrating cells were purified after isolation of pancreatic islets from collagenase P (Boehringer Mannheim, Laval, QC) digestion and centrifugation of the islets on a discontinuous Ficoll gradient. Free islets were hand-picked under a

dissecting microscope to a purity of ≥95%, and purified islets were cultured for 24 hr to allow the emigration of lymphocytes from the islets. After culture harvest and isolation of viable lymphocytes by density gradient centrifugation on Lympholyte-M (Cedarlane Laboratories, Hornby, ON), the cells were cultured for 48 hr with anti-CD3ε as above. Culture supernatants were assayed for their concentration of cytokines by ELISA. IL-4 levels were interpolated from a standard curve using recombinant mouse (rm) IL-4 captured by the BVD4-1D11 mAb and detected by the biotinylated BVD6-24G2 mAb while IFN-γ concentrations were measured using rmIFN-γ, the R4-6A2 mAb and biotinylated XMG1.2 mAb (all obtained from PharMingen, Mississauga, ON). Standard curves were linear in the range of 20-2000 pg/ml.

In some experiments, the relative levels of IL-2 and IL-4 secreted were quantified in a bioassay using the IL-2 dependent CTLL-2 T cell line (Gillis et al., 1977) and IL-4 dependent CT.4S T cell line (Li et al., 1989) (supplied by Dr. W. E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Two fold serial dilutions of test supernatants were added to CTLL-2 cells (1.5×10^4) and Ct.4S cells (5×10^3), which were cultured for 24 h and 48 h, respectively, in flat-bottomed 96 well-plates. Cell proliferation was assessed by addition of [^{3}H]-thymidine for 8 h prior to termination of culture, and [^{3}H]thymidine incorporation was determined as above.

Intrapancreatic Cytokine Analysis Intrapancreatic IL-4 and IFN-γ concentrations in tissue samples were quantified, as described in Chensue et al., 1992; and Lukacs et al,. 1994. Briefly, pancreata were isolated and snap frozen in liquid nitrogen. Upon analysis, the samples were homogenized and sonicated in protease inhibitor buffered cocktail followed by filtration

through 1.2 μm filters (Gelman Sciences, Ann Arbor, MI). The filtrates were analyzed for IL-4 and IFN-γ concentrations by ELISA, and the ELISA results were normalized relative to the total amount of protein per pancreas and recorded as ng/mg tissue.

GAD Antibody ELISAs The presence of anti-GAD antibodies in collected sera was determined by ELISA as previously described (Elliott et al., 1994). Briefly, sera samples were added at appropriate dilutions to plates coated with murine GAD_{67} (10 μg/ml). Using AP-conjugated goat anti-mouse isotype (IgG1 or IgG2a) antibodies with p-nitrophenylphosphate disodium in diethylamine buffer (substrate) the optical density was read at 405 μm to determine the relative amount of the individual anti-GAD isotype. All sera were titrated at 1:20, 1:40, 1:80, and 1:160 dilutions for anti-GAD_{67} antibodies. Since no significant differences were found between the IgG1 and IgG2a ratio at the 1:20 dilution between treated and untreated mice, all sera were tested for the specific isotypes (IgG1 and IgG2a) at the 1:20 dilutions.

Adoptive Cell Transfer Female NOD.Scid mice (n=5/group) 6 to 8 weeks of age were each injected i.p. with splenic T cells (10^7) from pre-diabetic female NOD mice previously treated with anti-CD28 mAb or control Ig. The recipients were followed for a maximum of 12 weeks after transfer and blood glucose levels (BGL) were monitored weekly.

Flow Cytometry Splenic T cells and thymocytes (10^5) were suspended in 0.1% BSA and PBS/0.001% NaN3, and were then incubated for 30 min at 4°C with various FITC- or PE-conjugated mAbs against different murine lymphocyte subpopulations and functional markers, including $CD3_{\varepsilon}$, CD4, CD8, CD19, CD25, CD69, CD44, L-selectin, CD40, LFA-1, B7-1 and B7-2 (PharMingen). Isotype matched (Ig)

antibodies were used as negative controls. Cell fluorescence was analyzed using a FACScan and Lysis II software (both from Becton-Dickinson, San Jose, CA).

Example 1 - Restoration of NOD T cell proliferative responsiveness by CD28 costimulation

Thymocytes and splenic T cells from 8 week-old NOD and control BALB/c mice were activated by plate-bound anti-CD3 in the absence or presence of varying dilutions (2 ng/ml - 2 μg/ml) of soluble anti-CD28 mAb. Cell proliferation was determined by [^{3}H]thymidine incorporation. The results are shown in Figures 1A and 1B. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

Figure 1A shows that CD28 costimulation provided by anti-CD28 markedly enhanced the anti-CD3-induced proliferative responses of NOD and BALB/c thymocytes, yielding 19.5- and 5.6-fold increases (at the highest concentration of anti-CD28) in these responses, respectively. Similar results were observed when an anti-TCRαβ mAb was substituted for anti-CD3 (data not shown). However, when quiescent NOD and BALB/c thymocytes were stimulated by anti-CD28 in the absence of anti-CD3 (or anti-TCRαβ), a low level of proliferation was observed which was equivalent to the basal proliferative response detected in the absence of any stimulus (data not shown).

Anti-CD28 mAb also significantly enhanced the NOD, and to a lesser extent the BALB/c, anti-CD3-induced splenic T cell proliferative response (Figure 1B). NOD and BALB/c splenic T cells were less responsive to CD28 costimulation (in terms of fold increases) than thymocytes from these mice, consistent with the notion that primed and naive T cells have different requirements for costimulation. Whereas primed splenic T cells

require only TCR engagement to proliferate and produce IL-2, naive thymocytes require at least one additional costimulatory signal for optimal proliferation.

NOD and BALB/c thymocytes obtained from 8 week old mice were activated by plate bound anti-CD3 in the absence or presence of 1 μg/ml soluble anti-CD28 mAb (optimal concentration). Culture supernatants were removed, diluted and assayed for their IL-2 and IL-4 content by stimulation of proliferation of the CTLL-2 and CT.4S T cell lines, respectively. The results are shown in Figure 1C and 1D. In Fig. 1C, the CTLL-2 cpm values of [^{3}H]thymidine incorporation for anti-CD3 activated NOD and BALC/c T cells represented by the highest supernatant dilution were 9,064 ± 1,246 and 3,715 ± 940, respectively. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of four different experiments.

Figure 1C demonstrates that anti-CD3 plus anti-CD28 costimulation significantly increased IL-2 production by both NOD (21.6-fold) and BALB/c (5.5-fold increase) but not BALB/c thymocytes (Figure 1D). This may be attributable to the higher basal level of IL-4 production by BALB/c T cells than NOD T cells. CD28 costimulation augmented the proliferative responsiveness, as well as IL-2 and IL-4 production, of NOD thymocytes to levels comparable to those of BALB/c thymocytes. This may occur by a CD28-mediated pathway that significantly enhances the differentiation and ability of NOD thymocytes to produce IL-4, which can subsequently stimulate T cell proliferation in an autocrine and/or paracrine fashion. The finding that IL-4 restores the proliferative responsiveness of NOD thymocytes by increasing their level of IL-2 production agrees closely with the reported role for IL-4 in the stimulation of IL-2 production by mouse T cells in response to plate-bound anti-CD3.

The data above suggest that the induction of NOD T cell responsiveness is dependent largely on the ability of IL-4 to increase IL-2 production and stimulate NOD T cell proliferation. These results also suggest that both NOD Th1 and Th2 cell proliferative responsiveness can be restored by CD28-mediated costimulation via a mechanism that is partially, if not primarily, dependent on the enhancement of IL-2 and IL-4 production, respectively.

Example 2 - Prevention of Insulitis by anti-CD28 Antibody

8 week-old and 25 week-old NOD mice ($n \geq 5$ in each group) were injected with either anti-CD28 mAb or control hamster Ig.

Following hematoxylin and eosin staining of pancreata, a minimum of 20 islets from each NOD mouse were observed and the degree of mononuclear cell infiltration was graded independently by two observers as follows: 0-normal; 1-peri-insulitis (mononuclear cells surrounding islets and ducts but not infiltrating the architecture); 2-moderate insulitis (mononuclear cells infiltrating <50% of the islet architecture); 3-severe insulitis (>50% of the islet tissue infiltrated by lymphocytes and/or loss of islet architecture).

Scores are shown in graphical form in Figure 2.

Anti-CD28 treatment of NOD mice during the inductive phase (2-4 weeks of age) of development of IDDM prevented destructive insulitis. At 8 weeks of age, these anti-CD28 treated NOD mice had 70% of healthy islets (insulitis score=0) as seen in Figure 2A.

At 25 weeks, in these anti-CD28 treated NOD mice (Figure 2B), the percentage of islets displaying severe insulitis (insulitis score=3) was considerably lower (19%) than that observed in control treated mice (46%), and anti-CD28 treated animals still possessed 22% of normal healthy islets (insulitis score=0) while normal islets were not present in the control animals. In

contrast, when anti-CD28 treatment was initiated after the onset of insulitis at 5 weeks of age, significantly less protection from insulitis was found (data not shown).

Example 3 - Prevention of autoimmune diabetes in NOC mice

Twenty female NOD prediabetic mice (randomized from five different litters) were injected three times weekly from 2 to 4 weeks of age with 50 μg of either the 37.51 anti-CD28 mAb or control hamster Ig, and then boosted at 6, 7 and 8 weeks of age. Another group of ten females (randomized from three different litters) were similarly treated from 5 to 7 weeks of age. Mice were screened weekly for the presence of hyperglycemia (BGL >11.1 mmol/L) starting at 8 weeks of age. Diabetes was diagnosed when mice were hyperglycemic for two consecutive readings. The results are shown in Figure 3.

Treatment of pre-diabetic NOD mice with anti-CD28 antibody at 2 to 4 weeks of age completely prevented the development of IDDM. At 28 weeks of age, 16 of 20 control mice had developed IDDM whereas none of 20 treated mice had developed IDDM (Figure 3A). If anti-CD28 antibody treatment was delayed until after 5 weeks of age, significantly less protection against IDDM was obtained (Figure 3B).

Anti-CD28 antibody treatment was unable to prevent cyclophosphamide-induced IDDM in NOD mice, regardless of whether cyclophosphamide was injected before or after anti-CD28 antibody administration (data not shown).

This results indicates that cyclophosphamide-sensitive regulatory T cells must be present and stimulated by anti-CD28 mAb in order to prevent IDDM by antibody treatment. Thus, CD28 costimulation represents a form of immunostimulation of NOD T cells which effectively protects against IDDM, particularly when anti-CD28 treatment is administered during the inductive phase of the disease.

Example 4 - Induction of IL-4 production *in vivo* by anti-CD28 antib dy treatment

Thymocytes, splenic T cells and islet infiltrating cells (10^6/ml) were pooled from at least 3 age-matched NOD mice at various times after treatment at 2-4 weeks with anti-CD28 mAb or control Ig, and were then stimulated with the 14.5-2C11 anti-CD3ε mAb (plate bound, 1/500 ascites dilution). After either 72 hr (thymocytes) or 48 hr (T cells and islet infiltrating cells) of culture, the concentration of IL-4 and IFN-γ in cell supernatants from triplicate cultures were determined by ELISA. The results are shown in Figure 4. Values shown are the mean ± SEM of three separate experiments.

Example 5 - Lack of enhancement of anti-CD3-stimulated T cell proliferation by treatment with anti-CD28 antibody

Thymocytes, splenic T cells and islet infiltrating cells ($2x10^5$/well) from 8 and 25 week-old NOD mice ($n \geq 3$) injected at 2 to 4 weeks with either anti-CD28 mAb or control hamster Ig were cultured in triplicate wells in the presence or absence of the plate-bound 145-2C11 anti-CD3ε mAb (1/1000 ascites) for 48 hr (T cells, infiltrating cells) or 72 hr (thymocytes). Cell proliferation was determined by [^{3}H]thymidine incorporation. Results are shown in Figure 5.

Example 6 - Pancreatic IL-4 and IFN-γ enhancement by anti-CD28 antibody treatment

NOD mice were treated with either anti-CD28 mAb (n=7) or control hamster Ig (n=5) at 2-4 weeks. Mice were sacrified at 25 weeks of age, and intrapancreatic IL-4 and IFN-γ concentrations were determined by ELISA. Results are shown in Figure 6A. Values were expressed as mean ng cytokine/mg tissue. Comparison between means was

performed by Student's *t* test, and a p value of <0.05 was chosen as the level of significance (**$p<0.001$).

Serum samples were assayed for anti-GAD antibodies as described above. Results are shown in Figure 6B.

Example 7 - Delay of IDDM onset by T cell transfer

Splenic T cells (10^7) from 25 week-old, pre-diabetic female NOD mice previously untreated or treated at 2-4 weeks with anti-CD28 mAb were injected into 6-8 week-old female NOD.Scid mice (n=5/group). The recipient NOD.Scid mice were followed for a maximum of 12 weeks after injection, and BGL were monitored weekly. Results are shown in Figure 7.

When splenic T cells from non-diabetic NOD mice (25 weeks of age) were transferred into NOD.Scid recipients, the transfer of IDDM was either prevented or significantly delayed if recipient mice received T cells from anti-CD28 treated donors rather than T cells from control Ig treated mice (Figure 7). All (5/5) of the mice injected with T cells from control Ig treated mice became diabetic between 35-40 days after transfer, while only 2/5 of the mice injected with T cells from anti-CD28 treated animals developed diabetes by 90 days post transfer.

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FIGURE 1

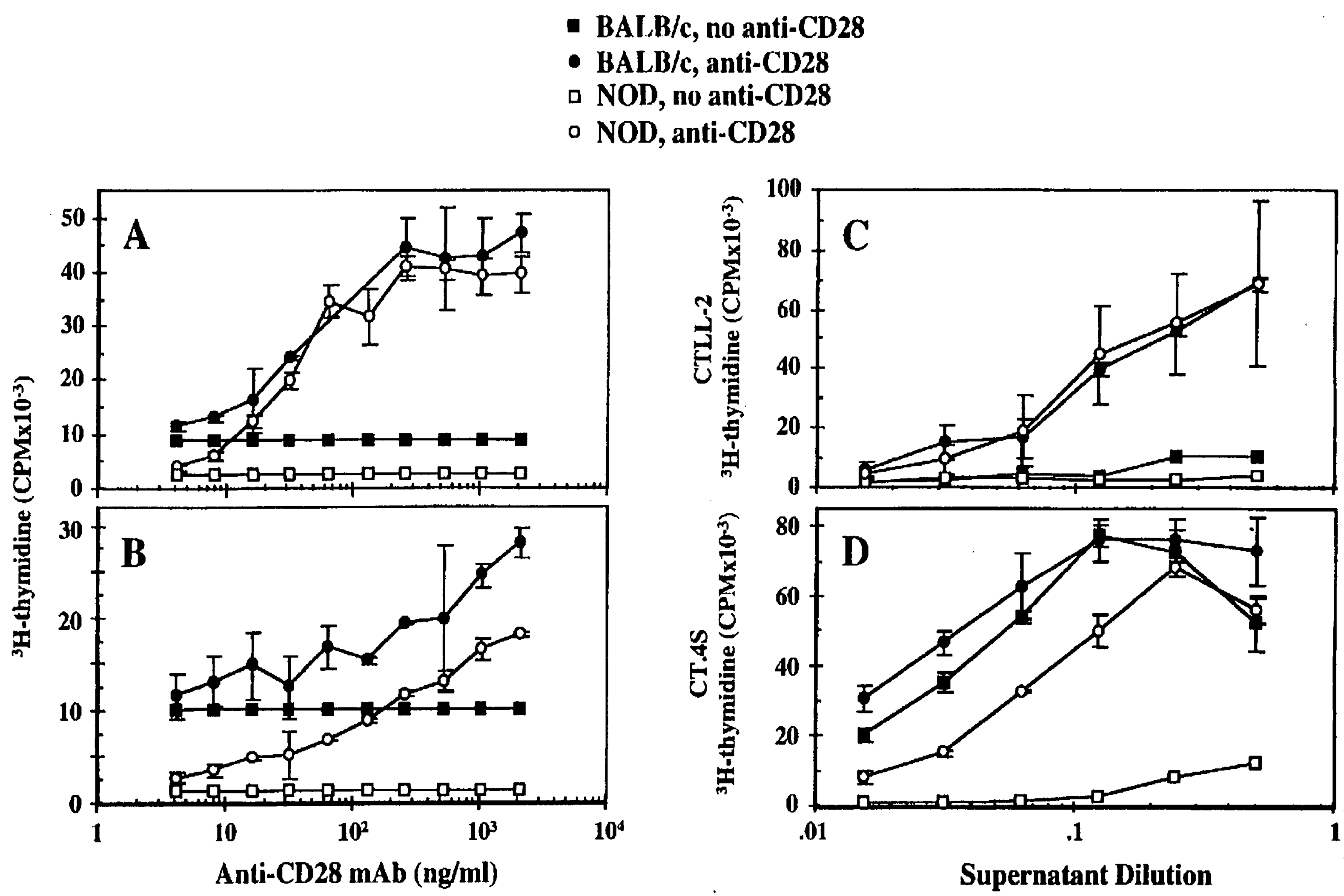

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FIGURE 2

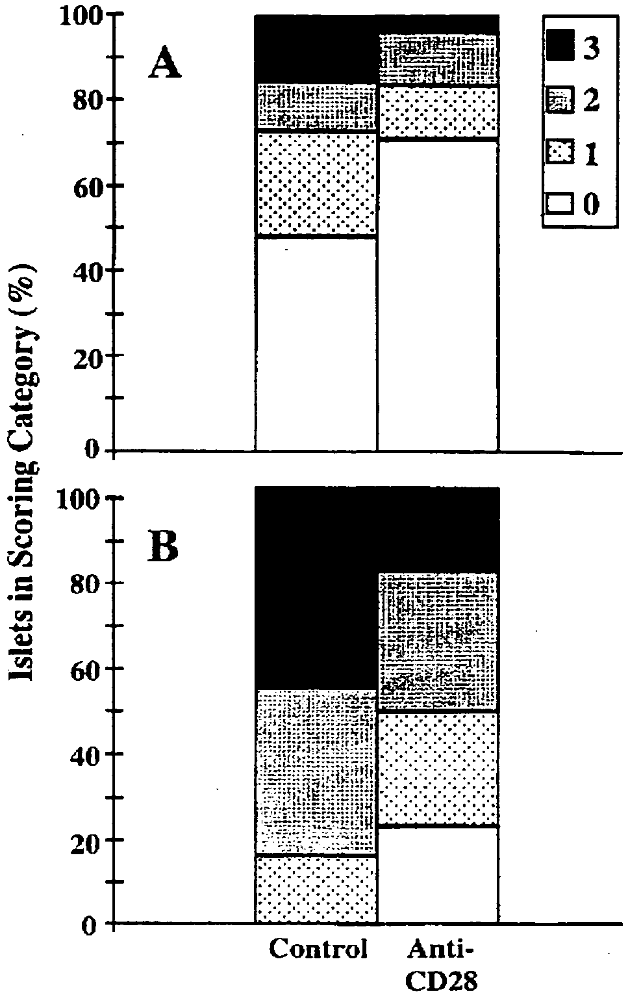

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FIGURE 3

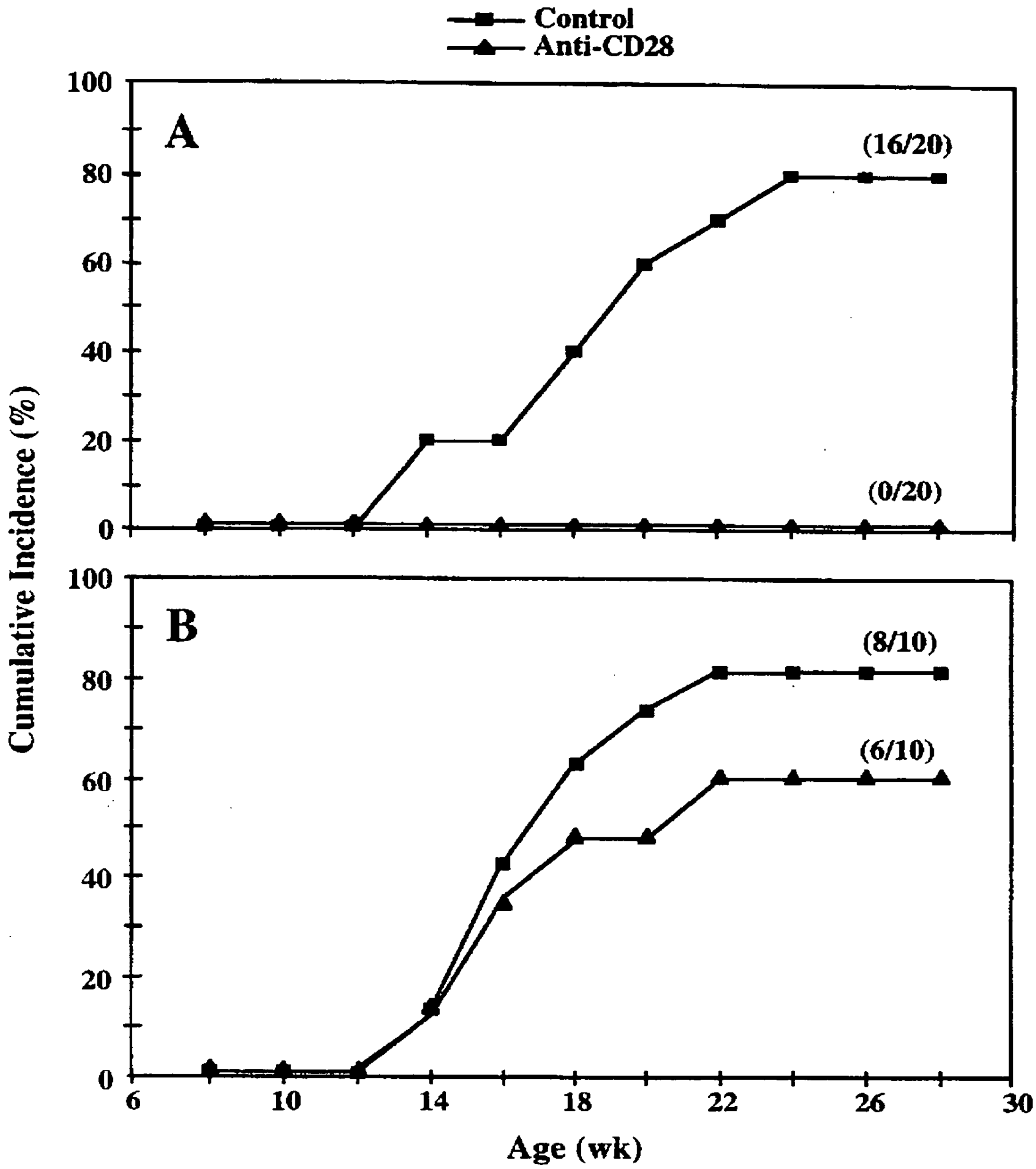

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FIGURE 4

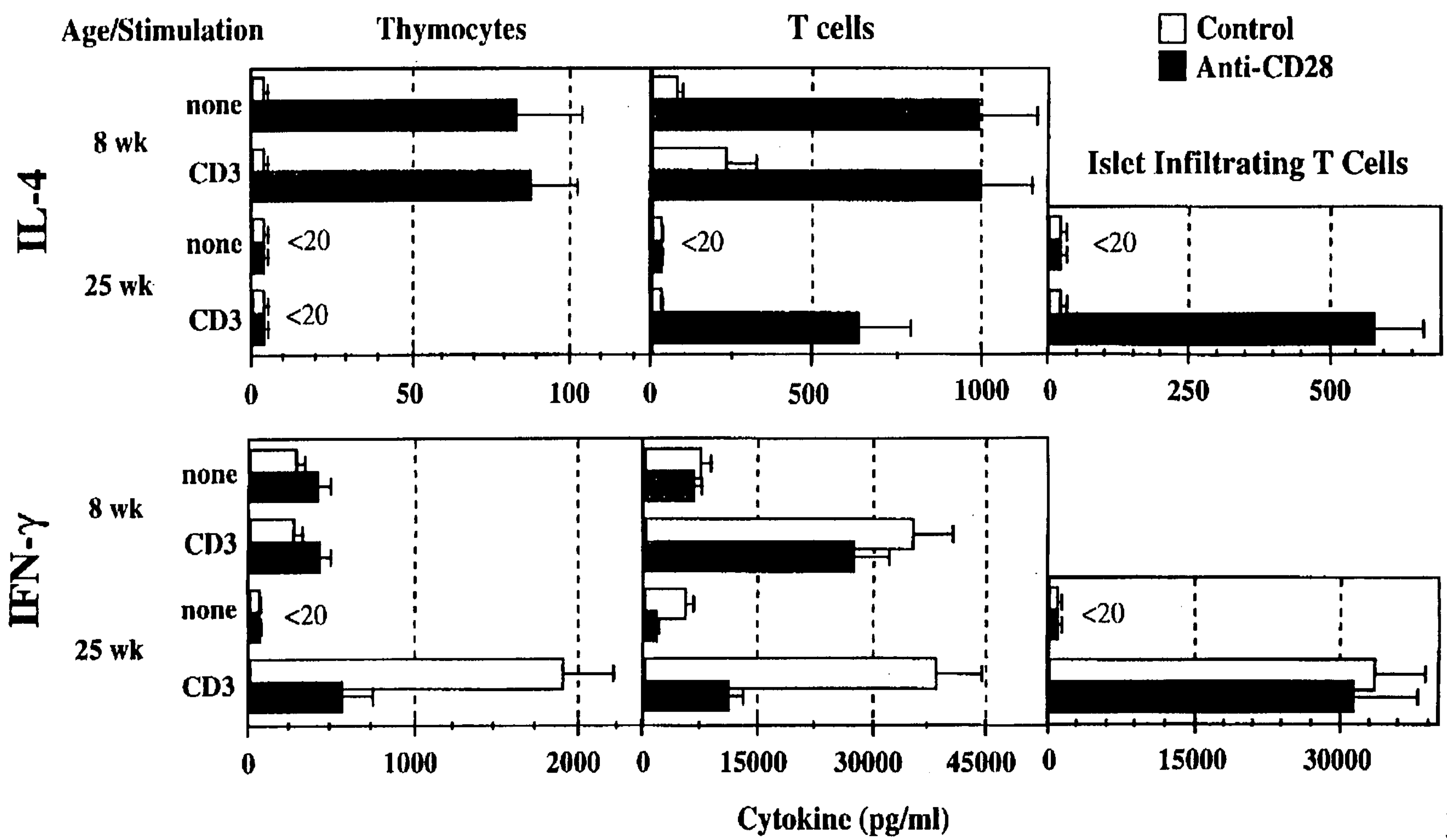
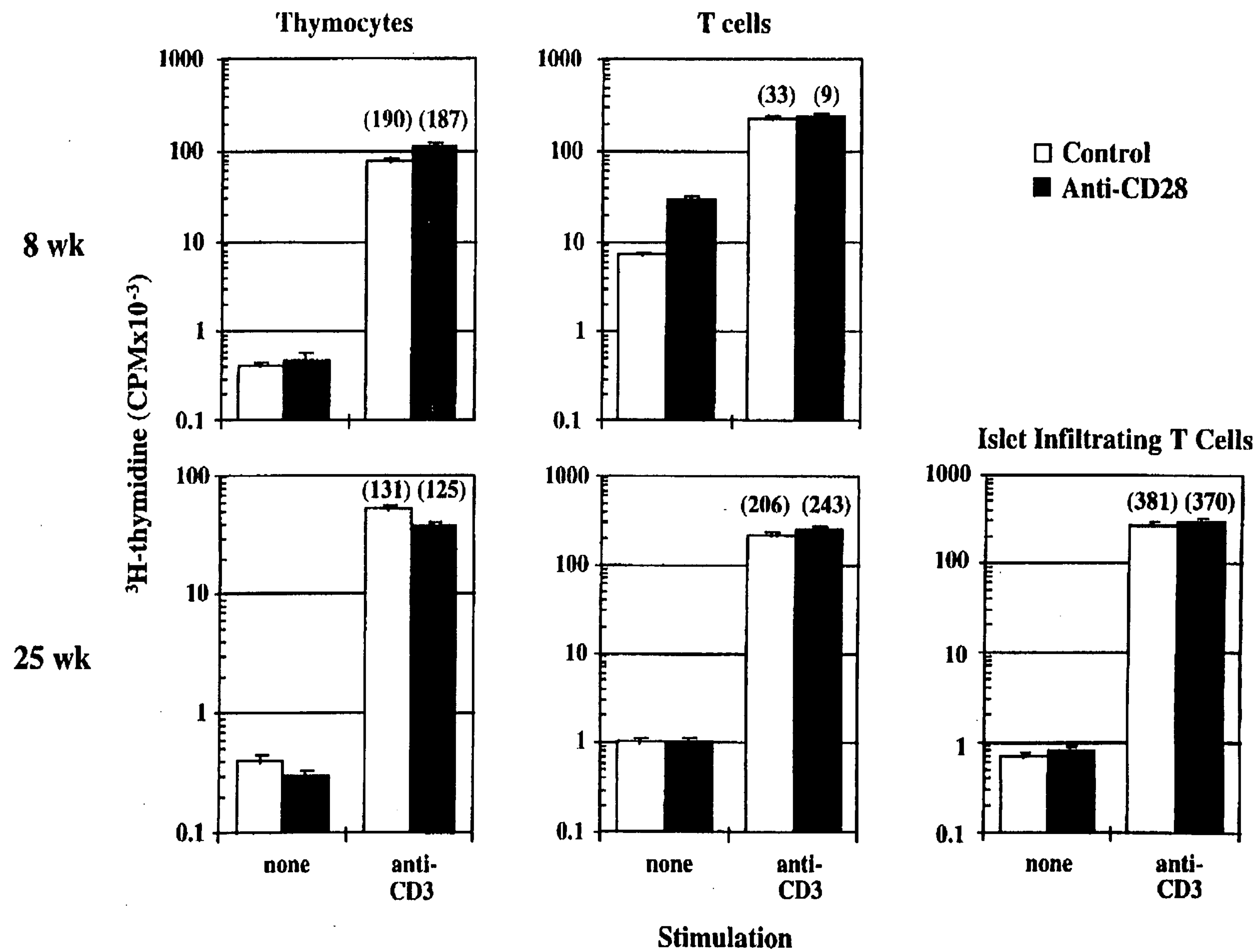


FIGURE 5



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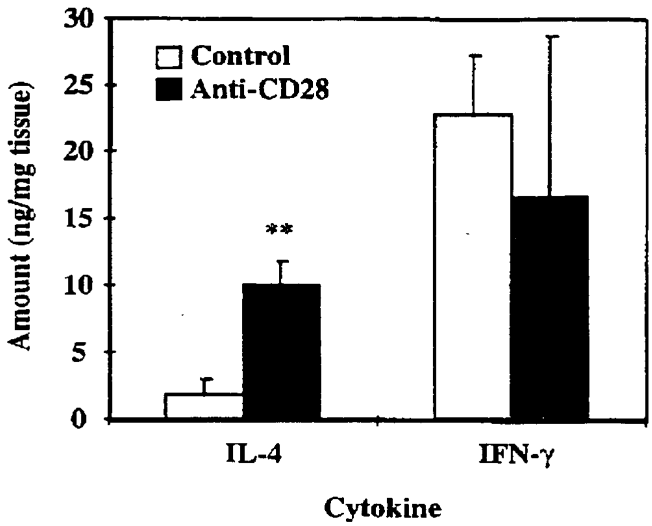
Control
Anti-CD28
Amount (ng/mg tissue)
30
25
20
15
10
5
0
**
IL-4
IFN-γ
Cytokine

2194814 FIGURE 6B

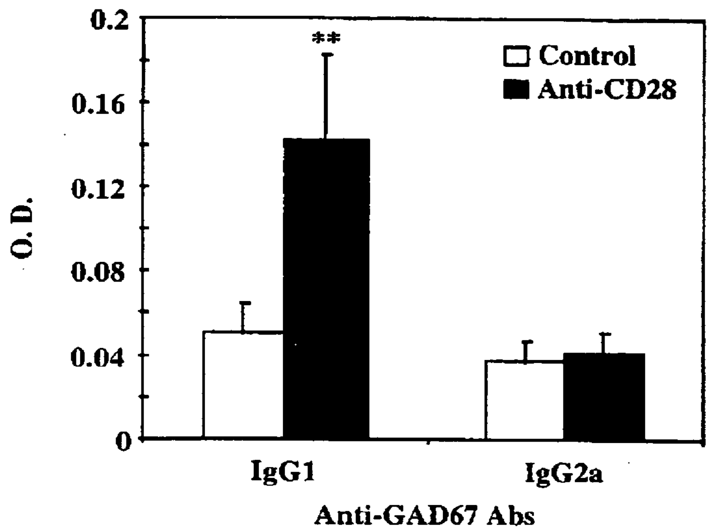

FIGURE 7

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